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Circulating RNAs in prostate cancer patients

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ABSTRACT

Growing bodies of evidence have demonstrated that the identification of prostate cancer (PCa) biomarkers in the patients' blood and urine may remarkably improve PCa diagnosis and progression monitoring. Among diverse cancer–derived circulating materials, extracellular RNA molecules (exRNAs) represent a compelling component to investigate cancer-related alterations.

Once outside the intracellular environment, exRNAs circulate in biofluids either in association with protein complexes or encapsulated inside extracellular vesicles (EVs). Notably, EV-associated RNAs (EV-RNAs) were used for the development of several assays (such as the FDA-approved Progensa Prostate Cancer Antigen 3 (PCA3 test) aiming at improving early PCa detection.

EV-RNAs encompass a mixture of species, including small non-coding RNAs (e.g. miRNA and circRNA), lncRNAs and mRNAs. Several methods have been proposed to isolate EVs and relevant RNAs, and to perform RNA-Seq studies to identify potential cancer biomarkers. However, EVs in the circulation of a cancer patient include a multitude of diverse populations that are released by both cancer and normal cells from different tissues, thereby leading to a heterogeneous EV-RNA-associated transcriptional signal. Decrypting the complexity of such a composite signal is nowadays the major challenge faced in the identification of specific tumor-associated RNAs. Multiple deconvolution algorithms have been proposed so far to infer the enrichment of cancer-specific signals from gene expression data. However, novel strategies for EVs sorting and sequencing of RNA associated to single EVs populations will remarkably facilitate the identification of cancer-related molecules.

Altogether, the studies summarized here demonstrate the high potential of using EV-RNA biomarkers in PCa and highlight the urgent need of improving technologies and computational approaches to characterize specific EVs populations and their relevant RNA cargo.

1. Introduction

Large-scale population screening and early detection of PCa represent the major defense against one of the most frequent causes of cancer-related deaths in men [1]. Early diagnosis and disease monitoring from early stages would enormously benefit from the identification of clinically relevant biomarkers (i.e. biomarkers associated with aggressive disease), which can be easily detected in blood or urine samples [2].

The blood-based test for the assessment of PSA (prostate – specific antigen) elevation is nowadays the most largely utilized approach for the detection of PCa [3]. However, PSA testing is limited in its ability to distinguish benign alterations of the prostatic epithelium, such as prostatitis or benign prostatic hyperplasia (BPH), from PCa [2]. Thus, additional circulating material released by cancer cells in the biofluids, such as circulating tumor cells (CTCs), cell free DNA (cfDNA) and RNA (cfRNA), and extracellular vesicles (EVs), are currently being explored as a potential source of PCa biomarkers [4]. The collection and analysis of circulating materials, generally referred to as 'liquid biopsy', is particularly relevant for solid cancers as it in principle offers the opportunity to assess the presence of malignant tissue via a blood drawn while limiting invasive procedures (i.e. needle biopsies) or high-resolution imaging modalities for body scan (i.e. **PET**: positron emission tomography; **CT**: computed tomography, **MRI**: magnetic resonance imaging) [5]. Compared to tissue biopsies, liquid biopsies not only represent a less invasive and minimally painful approach but also hold the potential to better capture the molecular heterogeneity of the tumor mass [6,7] and to detect molecular signal from metastases that might be difficult to biopsy (such as those in the bones). Liquid biopsy-based tests would be of particular relevance for PCa diagnosis to improve the limited specificity of the current PSA testing [4], while

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ideally focusing on the detection of high risk PCa, and to provide means for tracking potential independent clones during the treatment of metastatic patients.

Several recent studies demonstrated the utility of investigating cfDNA through next-generation sequencing (NGS) approaches to identify molecular alterations in advanced PCa patients [8,9]. For instance, by analyzing cfDNA in serial plasma samples it was possible to discover the emergence of specific genomic aberrations (such as AR amplification) in castration resistant PCa (CRPC) patients treated with anti-androgen therapy [10,11].

Analogously, isolation of CTCs from metastatic CRPC (mCRPC) and their subsequent enumeration and genomic characterization by NGS technologies contributed to the identification of biomarkers in specific clinical contexts, such as resistance to abiraterone and enzalutamide [12]. In particular, CTCs represent a valuable source not only for studying cancer-associated genomic alteration, but also to assess the expression of mutant variants, such as AR-V7 and TMPRSS2:ERG as well as of prostate specific mRNAs (e.g. KLK2, KLK3) [13,14].

Investigating cfDNA and/or CTCs as a source of diagnostic and predictive biomarkers in advanced PCa provide potential useful tools for clinical applications [15,16]. However, these circulating materials frequently pose a limit for early cancer detection and relevant diagnosis [16]. In this context, several efforts are currently ongoing to develop early diagnosis tests based on the detection of circulating RNA extracted from blood or urine [17]. For instance, the FDA approved a test, named Progensa Prostate Cancer Antigen 3 (PCA3), which is based on the detection of PSA and PCA3 mRNAs in the urine [18]. Additionally, the following tests are currently in course of validation: i) the ExoDx test (Prostate IntelliScore testing), which is based on the detection of ERG and PCA3 mRNAs extracted from urine-isolated exosomes [19]; ii) the SelectMDX assay that integrates urine-extracted HOX6 and DLX1 mRNA levels together with other risk factors [20].

Notably, analysis of circulating RNA has been recently combined with other circulating materials to develop multi-analyte liquid biopsies in cancer. For example, analysis of circulating RNA together with CTCs and cfDNA has been investigated for molecular profiling of cancer in mCRPC patients [21].

Altogether, these recently developed liquid biopsy-based tests highlight the significance and the promising potential of exploring cancer–associated circulating transcriptional information.

Here we summarize the types and relevant function of exRNAs circulating in the blood that may serve as useful PCa biomarkers for the future implementation of liquid biopsies in the clinic. Additionally, we highlight the challenges currently faced in EV-RNAs transcriptomics analysis mainly due to the difficulty to differentiate the RNA signal associated to cancer-versus normal cells-released RNA molecules.

2. Extracellular RNAs (exRNAs) encompass a large variety of species

During the last decade, several studies demonstrated the presence of RNA species that are released outside the cells, collectively indicated as ‘extracellular RNAs (exRNAs). ExRNAs were detected in the blood, the urine and the saliva, and represent potentially highly useful biomarkers for the development of liquid biopsies-based assays [22].

ExRNAs are protected from ubiquitous ribonucleases enzymes-mediated degradation through: *i)* the association with shielding carriers, such as lipoproteins (e.g. high-density (HDL) and low-density (LDL) lipoproteins) and proteins complexes (resulting in the formation of ribonucleoprotein complexes) [23], or *ii)* the encapsulation within the EVs [24].

Circulating EVs are lipid bilayer membrane-delimited nanoparticles naturally secreted by all types of cells into the extracellular spaces and into biofluids [25]. EVs comprehend a variety of subtypes including exosomes (50–200 nm), microvesicles (100 nm–1000 nm), apoptotic bodies (1000 nm-5000 nm) [26] and micron-sized particles named “oncosomes” (1–10 μm) [27]. Diverse molecules such as proteins, metabolites, lipids and nucleic acids are carried by EVs and potentially transferred between cells and the EVs cargo is indeed proposed for tracing malignant alterations back to tissues. Because of the overlapping physical features (dimensions and density), the cells-released EVs are hardly distinguishable from other lipidic components circulating in the biological fluids, such as HDL, LDL, chylomicrons and lipoproteins’ aggregates [26]. To this purpose, specific methods such as ultracentrifugation and size – exclusion chromatography (SEC) are required to isolate EVs from fluids before purification of molecular components from their core [28]. The solely presence of circulating EVs combined with other circulating tumor derived–materials, such as CTCs, has been proven predictive of overall survival (OS) in CRPC, metastatic colorectal cancer (mCRC), metastatic breast cancer (MBC) and non-small cell lung cancer (NSCLC) [29].

Besides their association with proteins or EVs, the exRNAs are frequently generically referred to as “circulating RNA” or cfRNA. Initially, circulating RNAs were essentially investigated as cell free nucleic acids [30], and EVs isolation approaches and EVs RNA cargo have only recently emerged in the biomedical research. When the isolation of EVs or exosomes from biological fluids is not performed for RNA detection, the circulating RNA is usually referred as cfRNA [31]. When reviewing the literature, it is always a good practice to check the methods of the studies as some confusion on the nomenclature of exRNAs is not rare.

Levels of cfRNA were observed being stable in plasma, with little or no variation to natural diurnal cycles and meal consumption [32], suggesting cfRNA as an ideal circulating material for liquid biopsies [24]. Albeit cfRNA still lack standardization of protocols for extraction, detection and validation [32,33], diverse species of cfRNA have been identified and proposed as potential biomarkers candidates [34,35].

Indeed, cfRNA has already been demonstrated as a potential source of tumor-associated biomarkers in several biofluids and cancer types, for example: 1) non-coding cfRNA have been identified in the plasma and saliva of head and neck cancer patients [36]; 2) urinary UBE2C cfRNA levels were proposed to discriminate between bladder cancer and healthy controls [37]; 3) fragments of VEGF mRNA were detected in urine samples of renal cell carcinoma patients and investigated as a potential molecular marker for diagnosis [38]. cfRNA as material for the detection of circulating tumor biomarkers has been reported also for mCRPC patients where androgen receptor aberrations (AR mutant variants such as ARV7, ARV9, copy number variation, somatic mutations) were detected on plasma cfRNA [39].

Since 2007 several research studies, such as those conducted by Valadi H. et al. and Skog J. et al. demonstrated the presence of mRNAs and miRNAs inside the EVs [40,41]. Since then, the EVs and their RNA cargo emerged as an attractive circulating source of exRNAs in normal and pathological conditions.

Several aspects rapidly made EVs an appealing target for the characterization of exRNAs, including: 1) the EVs lipidic bilayer membrane constitute an ideal carrier unit to safely transport multiple RNAs; 2) exRNAs encapsulated into the EVs are separated from other materials circulating in the body fluids; 3) the EVs are released by all cell types and their RNA cargo represents an informative portion of the total cellular RNA; 4) the development of NGS technologies allowed to sequence and to profile minimal amounts of EVs-derived RNAs (EV-RNAs); 5) the rising hypothesis that exRNAs might play a role in many intercellular communication processes, such as cancer metastasis [42,43]. Finally, diverse studies on EVs revealed an increased quantity of circulating EVs in the blood and the urine of patients affected by malignant diseases, including PCa [44,45].

Altogether this has resulted in the common association of exRNAs with EVs–carried RNAs (EV-RNAs), especially in the field of liquid biopsies [33,46].

Many efforts have been deployed to set up and constantly improve methods for the purification and the sequencing of exRNAs associated to

diverse classes of EVs [47,48].

Importantly, the recent literature on the transcriptional information carried by circulating EVs demonstrated that several species of RNAs are embedded inside the EVs. The EV-RNAs cargo is constituted by RNAs fragments of diverse dimensions (generally small fragments with a peak size around 200 bp) and include: mRNAs, precursors pre-miRNAs and mature miRNAs, snoRNAs, rRNAs, tRNAs, lncRNAs, pi-RNAs, Y-RNAs, vtRNAs and mitochondrial RNAs [46].

Here we review EV–associated mRNAs, miRNA, lncRNAs, and circRNAs that have been most widely associated with tumor progression, metastasis, and resistance to therapies in PCa.

- **mRNA**

Fragments or full -length mRNAs have been reported to be associated to EVs released by cancer cells, including those from PCa [46]. The mRNA cargo of EVs isolated from the urine or the blood has already been validated as a source of prostate–specific mRNAs, such as the transcript encoding for the proto-oncogene ERG (EXO106 score) [19] or the oncogenic fusion gene TMPRSS2:ERG [49,50]. In this respect, Leyten G. H. et al. showed the presence of the TMPRSS2:ERG transcript in urinary exosomes and reported a significant PCa detection predictive value when combined with ERSPC (European Randomised Study of Screening for Prostate Cancer) risk calculator, thereby demonstrating the prognostic value of TMPRSS2:ERG transcript and its potential for limiting the number of unneeded prostate biopsies [51]. Further studies demonstrated the value of combining urinary TMPRSS2:ERG transcript with other markers, such as the PCA3 and the PSA score [52].

Additional mRNAs, such as those associated with neuroendocrine (NE) features (e.g. BRN2 and BRN4) of PCa have been found in bloodstream circulating EVs [53]. Bhagirath D. et al. demonstrated that serum-derived EVs carry NE markers, such as BRN4 and BRN2, whose levels were shown by PCR analysis to be increased in CRPC patients with an NE phenotype compared to CRPC patients with adenocarcinoma features [53]. Additionally, blood-derived EVs were useful to detect the expression of the splice variant AR-V7 in metastatic patients to predict the emergence of resistance mechanisms to hormonal treatments [54,55]. Blood cfRNA levels of both AR and AR-V7 have recently been utilized in multiparametric liquid biopsies to follow mCRPC during time and at progression upon the use of specific treatments such as PARP-inhibitor [56]. In another study, Fettke et al. integrated expression of circulating AR and its variants (AR-V7 and AR-V9) in a cohort of mCRPC patients undergoing treatment with AR pathway inhibitors or chemotherapy to associate the presence of AR (and/or its analyzed variants) with poor outcome [39]. Furthermore, AR full length and AR-V7 mRNAs were detected in urinary EVs collected from CRPC patients, thereby demonstrating the concrete possibility of using the detection of such transcripts in both urine and blood liquid biopsies [57].

Despite the functional activity that circulating mRNAs might play inside recipient cells or within biofluids, all together these numerous findings confirm mRNAs cargo of EVs as a compelling source of prostate cancer biomarkers.

- **miRNAs.**

The miRNAs family represents one of the most observed RNA classes associated to EVs, likely because miRNA is one of the RNA types more frequently profiled by transcriptional analysis [58]. Attention to miRNAs as circulating RNA species transported by EVs [59] or protein aggregates [60] has also been promoted by the observation that extracellular miRNAs (i.e. miR-18a, miR-21, miR-155, miR-221, and miR-375) are deregulated in association with many cancer types including PCa [61].

In PCa patients, specific miRNAs have been identified in urine, plasma or serum, even though the biogenesis of such circulating miRNAs has not been fully elucidated yet [62,63]. Few potential mechanisms have been proposed so far for miRNAs sorting into EVs, including: 1) a pathway involving the neural sphingomyelinase 2 (nSMase2); 2) a pathway related to the miRISC (miRNA induced silencing complex); 3) a specific miRNA motif (GGAG in 3') that is recognized by the sumoylated heterogeneous nuclear ribonucleoproteins (hnRNP); and 4) other specific miRNAs sequences [64]. The sorting of miRNAs into exosomes is probably a selective and active process where RNA binding proteins and membrane associated proteins are considered important for active encapsulation of specific miRNAs [65].

To explore the miRNAs circulating in the blood as potential biomarkers in PCa, studies were conducted on *in silico* available RNA data sets and/or on RNAs isolated from body fluids such as plasma and serum. For instance, Souza M.F. et al. [66] conducted an *in silico* analysis on the TCGA database followed by RT-qPCR validation on plasma samples of 102 untreated PCa patients and 50 control individuals leading to the nomination of the expression of miR-200b and miR-200c as significantly associated with PCa. Specifically, miR-200b showed association with PSA >10 ng/mL and bone metastasis, while miR-200c expression was correlated with Gleason score [66]. Of relevance, these two miRNAs were also reported as critical regulators of PRKAR2B expression, a molecular factor implicated in PCa progression [67]. Interestingly, recent research from Gandellini P. et al. [68] demonstrated a signature of three miRNAs (miR-511–5p, miR-598–3p, miR-199a-5p) as a promising biomarker to improve active surveillance for low risk PCa.

Additional evidence of miRNAs as potential biomarkers in PCa patients liquid biopsies include miR-20a that was both characterized for its role in PCa tissue and further detected in the circulation of patients. Specifically, the miR-20a was found to be up-regulated in PCa tissue and established as a promoter of prostate cell invasion and migration by targeting ABL2, an important factor involved in several cancer types [69]. Shen J. et al. [70] detected overexpression of miR-20a in plasma samples of advanced PCa patients. Consistently, Mohammadi Torbati P. et al. showed up-regulation of circulating miR-20a by qRT-PCR on 40 PCa patients serum samples before prostatectomy compared to healthy controls [71].

Another example of miRNA proposed as circulating biomarker is represented by miR-145. The miR-145 was found altered in several types of cancers and its alterations were also studied in PCa where low expression levels were proposed as a predictor of poor prognosis [72]. More recently, qRT-PCR studies on PCa and normal prostatic tissues associated miR-145 to a set of miRNAs (including miR-221 and let-7c) under-expressed in low risk PCa [73]. Evidence for circulating miR-145 have been reported by qRT-PCR on exosomes isolated from urine samples of PCa where its expression levels resulted upregulated compared to BPH and healthy controls [74]. Expression of miR-145 was also reported on exosomes isolated from blood samples of PCa patients after radiation therapy, even if a clear variation of its expression level in the circulation was not established when compared to relative controls [75].

Among other miRNAs, the miR-221 has been investigated as potential circulating biomarker for solid cancers [76,77] including PCa [78]. Plasma levels of miR-221 in combination with Prostate Health Index (PHI) were proposed by Ibrahim N.H. et al. as diagnostic and prognostic marker in PCa [79]. The importance for the identification of this specific miRNA in circulation is due to its very well characterized role as onco-miRNA in prostate [80]. Specifically, the downregulation of miR-221 in prostate tissues was correlated with Gleason score, tumor progression and recurrence in high risk PCa patients [81]. *In vitro* and *in vivo* experiments proved miR-221–5p as a tumor suppressor, whereby increased levels of miR-221 were effective in limiting migration and extravasation of PCa cells in animal models. Conversely, downregulated levels were associated with metastasis or primary tumor compared to normal prostate tissue [82].

So far, a series of miRNAs embedded in blood-derived exosomes have

been correlated to PCa, including 1) increased levels of miR-1246 were observed in serum isolated exosomes of PCa patients and associated to aggressive PCa [83]; 2) miR200c-3p and miR-21–5p were found more expressed in PCa than in BPH (Benign Prostatic Hyperplasia) patients, while Let-7a-5p showed diverse levels depending on the Gleason score [84]; 3) miR-290 and miR-375 were shown to be upregulated in CRPC patients and significantly associated with overall survival rate [85]; 4) oncogenic miR-424 recently was associated with aggressive PCa [86]; 5) plasma exosomal miR-423–3p was proposed as a potential predictive biomarker of CRPC [87]; 6) combination of miR-17–3p and miR-1185-2-3-p were recently used by Urabe F. et al. to build a diagnostic model for PCa [88].

Interestingly, increased levels of miR-141 and miR-375, two of the most generally investigated miRNAs, were identified by multiple studies in both urine- and plasma-derived exosomes of PCa patients [89–91]. An additional miRNA, miR19b, was detected with remarkable high specificity and sensitivity by Bryzgunova O.E. et al. in the exosome–enriched subfraction of PCa patients' urine samples [92].

In addition to specific single onco-miRNAs, sets of miRNAs have been suggested as potential circulating biomarkers in PCa. For example, the miR-20a with miR-21, miR-145 and miR-221 set was found associated to low and high risk PCa patients [70]. Similarly, a four-miRNAs signature composed of miR-17, miR-20a, miR-20b and miR-106a identified from blood samples was proposed for PCa patients to predict risk stratification after radical prostatectomy [93]. A variation in the serum expression levels of a set of four circulating miRNAs (miR-141, miR-182, miR-200b, and miR-375) was detected in correlation with total PSA (TPSA) [94] and proposed as diagnostic biomarker to differentiate between BPH and PCa.

Current research work is investigating circulating miRNAs in combination with other tumor-derived materials detectable in the blood or in the urine such as CTCs [95,96]. Cheng H.H. et al. reported a positive correlation between miR-141, miR-200a, miR-375 and CTCs in baseline plasma of metastatic hormone sensitive PCa patients. Importantly, they demonstrated that levels of miR-375 and CTCs counts equally predicted 28-week PSA response to androgen deprivation therapy alone or in combination with cituxumumab [97].

Despite all these pieces of evidence, as of today there is no molecular miRNA-based assay utilized in the clinic for urine or plasma samples from PCa patients.

- **lncRNAs**

PCA3 represents the most studied lncRNA in prostate [98]. Specifically, this lnRNA was utilized to develop a urine test [99], which was approved by FDA for use in the clinic [100]. In addition to PCA3, PCa-associated circulating lncRNA include MALAT1 in plasma as well as FR0348383, SCHLAP1 and lincRNA-p21, which are exosomal RNAs isolated from post – DRE (digital rectal exam) in the urine [101].

High plasma abundance of MALAT1 have been reported as critical to discriminate between BPH and PCa [102]. Additionally, MALAT1 levels were shown to decrease 7 days after surgery in 9 out of 10 patients, thereby providing supporting evidence for its potential role as circulating biomarker of advanced PCa. Furthermore, assessments conducted on urine samples showed that MALAT1 RNA significantly correlated with PCa detection [102].

Despite being less investigated than MALAT1, lncRNA FR0348383 in the urine might represent an attractive biomarker for PCa diagnosis to avoid unnecessary tissue biopsies [103]. However, additional analytical and clinical validation are still needed before considering lncRNA FR0348383 as a biomarker. Similarly, SChLAP1 levels in post-DRE urine samples were correlated with Gleason score, although also in this case further characterization is needed for biomarker classification [104].

Although significantly upregulated in PCa compared to BPH, the lincRNA-p21 failed to score as a biomarker because of its very low circulating levels [105].

Taking into account the latest progresses in NGS technologies and ultrasensitive methods (such as ddPCR) for the detection of RNAs low amount, it is very likely that EVs-associated lncRNAs as well as fragments of mRNAs will be routinely analyzed in liquid biopsies in the next future.

- **circRNA (circular RNAs)**

The circRNAs are considered to be robust RNA molecules because of their intrinsic structure. circRNAs lack free ends and are, therefore, less exposed to exonucleases-mediated degradation [106]. Notably, circRNAs were found in EVs [107,108]. For example, increased circulating exosomal circMYC was detected in patients with nasopharyngeal carcinoma and correlated with clinical variables, such as tumor size, metastasis and overall survival [109]. At the present, it is known that circ-RNAs function as microRNAs sponges [110], however their role in cancer (including their function inside EVs) has not completely been elucidated yet. Specific functional mechanisms have been recently described for the exosomal circ-SLC19A1 in PCa [111]. Zheng Y. et al. reported that PCa cells can regulate cell growth and invasion through the miR-497/septin 2 pathway by loading EVs with high levels of circ-SLC19A1 [112].

Despite the abundance of circRNAs into EVs, the clinical validation of this RNA class in liquid biopsies for PCa diagnosis and prognosis remains to be established.

3. Challenges in exRNAs transcriptomics analysis

3.1. Challenges associated to diverse methods for EVs isolation

Last few years have witnessed the development of a multitude of EVs-isolation methods that are characterized by different approaches, efficiency and time requirements [113,114]. Several studies have compared these methods but a gold standard is not been established yet [28]. Despite all the differences and peculiarities, these methods allow EV-RNAs recovery and quality which are suitable for the downstream analysis by sequencing [115]. Importantly, results may change dramatically on the basis of the used EVs isolation method; therefore, biological data interpretation needs to take into account this layer of complexity. It is known that different EVs isolation methods can lead to EVs mechanical stress, such as that observed upon EVs isolation by ultracentrifugation [116], potentially resulting in erroneous data interpretations. Furthermore, the EVs isolation method can directly impact on the enrichment for specific EVs subpopulations, each of them containing a characteristic repertoire of RNAs [117]. For instance, RNA profiles showed that rRNAs were primarily detectable in apoptotic bodies, while smaller RNAs (free of ribosomal RNA peaks) were more present in exosomes. In contrast, microvesicles contained little or no RNA [118].

3.2. Challenges associated to diverse methods and protocols for purification of exRNAs

Specifically, while studying EVs transcriptional cargo, both EVs and RNA isolation steps need to be taken into careful consideration. A recent paper compared five different isolation methods and their suitability for miRNA-based biomarker discovery [115]. These data showed that precipitation and membrane affinity-based EVs isolation methods are specifically suitable for small RNA sequencing and miRNAs-based patient classification. Their conclusions are restricted to the comparison and differential expression analysis of a specific RNA biotype (miRNAs) in serum-derived EVs from patients with sepsis and from healthy volunteers. Additionally, they demonstrated that the specific isolation method associates with non-EV-derived protein contamination (assessed as ratios between NTA particle counts and proteins concentration) and with different EVs size distributions [115], which suggests at least a partial

preferential isolation of EVs sub-populations.

In another work [119], a similar approach was used to compare EVs-miRNAs from PCa patients' and healthy volunteers' semen [119]. In that case, the miRCURY Exosome kit for EVs-isolation showed the highest particle recovery but a low RNA purity. Regardless of the latter aspect, the recovered RNA was suitable for subsequent miRNA analysis [119]. Interestingly, in the same study it was also reported a significant over-representation of specific miRNAs, such as miR-663b, in EVs extracted by ExoGAG, thus hypothesizing a selective isolation of specific miR-663b-containing EVs populations through that EVs isolation kit [119].

Other studies [120,121] showed an overall correlation between miRNAs obtained through different EVs isolation methods and significant differences upon analysis of specific transcripts. In this case it was found a dramatic difference between isolation methods when looking at different small RNAs biotypes, with a landscape dominated by rRNA or tRNA dependently on the chosen method.

Additionally, significant differences in the RNA yield, purity and size have to be taken into account when considering different RNA isolation methods [122].

Overall, regardless of the EVs isolation method, EV-RNAs represent a valuable source of information in cancer patient liquid biopsies [123]. In this respect, the direct comparison of healthy donors' and cancer patients' EV-RNAs-derived transcriptome represents a feasible and promising approach.

3.3. Challenges associated to lack of standardized pipelines/protocols for computational analysis of exRNAs

Unfortunately, the lack of a gold standard EVs isolation method and of a general consensus in the field about RNA cargos of EVs subpopulations make the comparisons of transcriptomics data from different studies quite difficult. Similar difficulties are encountered also for the analysis of circulating RNAs, not associated to EVs, due to lack of standardized computational methods. Several online resources aim to collect, organize and homogenize data from different sources (Table 1) with the goal of establishing a common ground for EV-RNAs and exRNAs not associated to EVs high-throughput analyses. Specific pipelines (mainly focused on short RNA [124]) have been proposed but, given the EVs' and relevant RNA cargo's heterogeneity, a one-size-fit-all solution may be sub-optimal. Recently, a total RNA sequencing approach has been tested on different biofluids [47] highlighting marked differences in reads distribution across sample types.

A standardized EV-RNAs sequencing data computational analysis would be beneficial to the field. However, the biological variability and the current lack of specific EVs populations' characterization make the RNA-seq analysis standardization quite difficult to achieve. These aspects might also explain the inconsistencies across different studies that have been observed so far [125].

Notably, a recent study analyzed EVs-small RNA data from multiple publications and diverse biofluids, by using the same computational pipeline [125]. The study reported that miRNA expression profiles primarily cluster by study, despite of the uniform computational processing. This observation supports the hypothesis that a potential source of variation may be represented by the presence of different exRNAs carriers (i.e. EVs, lipoproteins, etc), each one of them having a characteristic cargo profile and differential enrichment in a specific biofluid [125]. In particular, the study identified specific clusters of molecules, differentially associated with low-density vesicles (LDV), lipoproteins and AGO2-ribonucleoproteins.

LDV and high density vesicles (HDV) associated exRNAs were previously characterized in mast cells [126]. It was observed that, while both LD and HD fractions contain miRNAs and mRNAs, HD exRNAs are enriched in lincRNA, antisense RNA, vault RNA, snoRNA, and snRNA [126].

The heterogeneity of RNA species detected in different EVs populations uncovers possible mechanisms of selective sorting [127,128]. In this respect, several groups have already identified a series of miRNA sorting mechanisms, such as those involving the binding to RNA-binding proteins and membrane proteins [65].

For instance, the presence of circulating Ago2-miRNA complexes in human plasma suggested that Ago2 might play a role in stabilizing secreted miRNAs [129] and might be implicated in miRNAs binding and sorting into EVs through the KRAS-MEK-ERK signaling pathway [130]. Other potential candidates for active miRNAs sorting mechanisms are Major Vault Protein (MVP) [131], Y-Box Binding Protein 1 (YBX-1) [132], and Caveolin-1 [133].

Notably, post-transcriptional modifications, such as 3' end urydilation, have been associated with small RNA secretion via exosomes [134].

Concerning longer transcripts, reads from EVs-mRNAs showed abundant depth of sequencing across introns, suggesting the export of either unprocessed unspliced mRNA transcripts or, more likely, RNA fragments [128].

Finally, although lncRNAs and circRNAs are expressed at low levels in cells, several studies have shown that non-coding transcripts are enriched in EVs, even when they are down-regulated in the parental cells, thus suggesting a selective sorting into EVs [128,135–137]. Despite the amount of evidence associating EVs population to their RNA cargo, a detailed and broadly accepted classification of specific RNA biotypes in EVs subpopulation is lacking.

Upon EVs analysis from cancer patients' liquid biopsies, the heterogeneity of EVs populations, of their cellular sources and of specific RNAs cargos represents an important layer of complexity.

Specifically, besides cancer EVs, in each sample the majority of the EVs derive from normal cells of different tissues. Additionally, some EVs may derive from not transformed cells that play a key role in cancer progression and metastasis formation, such as tumor microenvironment cells, metastasis niches and immune cells (**see paragraph 2**). Since no single-EV sequencing approach has been reported yet, nowadays EV-RNAs transcriptomics analyses rely on pure computational approaches to disentangle the aforementioned complexity and heterogeneity of the signal.

As the gene expression profile of an admixture of cells is the result of a linear combination of the genes specifically enriched in each cell type [138], several deconvolution algorithms have been proposed to infer the enrichment heterogeneity of cell types from the gene expression data [139–142]. Although these algorithms have been successfully used to trace the fraction of immune cells from tissue transcriptional data, they are not directly applicable to EVs transcriptomics data. More recently, few attempts were made to deconvolve EVs transcriptomics populations from liquid biopsies [143,144]. The EV-Origin algorithm has been used to predict the relative enrichment of hemopoietic cells and solid tissues from exLR-profiles [143,144] comparing different disease conditions (in this specific case prostate tissue was excluded to avoid any gender-related bias). In a recent work on melanoma, researchers created a Bayesian probabilistic deconvolution model to estimate the contribution from tumoral and non-tumoral sources [144]. The method is computationally intensive and can run on a single or few genes, therefore a simplified multi-gene version has been proposed. However, this approach has proven to be able to discriminate specifically tumor and immune components with no capability to dissect the diverse EVs population contributing to cancer mechanisms, such as metastasis formation and tumoral microenvironment regulation (**see paragraph 2**).

A further source of noise in EVs transcriptional data is represented by the inter-patient variability. Recent studies identified EVs as key players within the secretome of senescent cells and showed their direct involvement in senescence-associated secretory phenotype (SASP) [145]. Furthermore, EVs have been linked to cardiac [146,147] and neurodegenerative diseases [148,149] thereby suggesting that the cancer signals can be overshadowed by the overall health status of a patient. Conveniently, liquid biopsy allows serial sampling from the same

Table 1
Online resources relevant to EV-RNAs high-throughput analyses.

Name	short description	first year of publication	molecules	type of data	publication	site
ExoCarta	Manually curated Web-based compendium of exosomal proteins, RNAs and lipids.	2012	mRNA, miRNA, proteins and lipids	annotation of molecules and experiments	https://doi.org/10.1016/j.jmb.2015.09.019	http://www.exocarta.org/
exrna data portal	Repository that includes small RNA sequencing and qPCR-derived exRNA profiles from human and mouse biofluids. All RNA-seq datasets are processed using the exceRpt small RNA-seq pipeline (Rozowsky et al., 2019) and ERCC-developed quality metrics are uniformly applied to these datasets.	2019	small ncRNAs	high-throughput datasets and uniformed quality metrics	https://doi.org/10.1016/j.cell.2019.02.018	http://exrna-atlas.org/
Vesiclepedia	Manually curated compendium of molecular data identified in different classes of EVs among different species. Last udate in 2019	2012	lipid, RNA and protein	molecular annotation	https://doi.org/10.1371/journal.pbio.1001450	http://www.microvesicles.org/
Urinary Exosome Protein Database	Based on published protein mass spectrometry data from the NHLBI Epithelial Systems Biology Laboratory (ESBL). All data are from urinary exosomes isolated from healthy human volunteers.	2009	proteins	proteins annotation	https://doi.org/10.1073/pnas.0403453101 https://doi.org/10.1681/ASN.2008040406	https://hpcwebapps.cit.nih.gov/ESBL/Database/Exosome/
miRBase	Website that provides a wide-range of information on microRNAs, including their sequences, their biogenesis precursors, genome coordinates and context, literature references, deep sequencing expression data and community-driven annotation. miRBase also acts as a portal for third party information about microRNA genes and sequence, linking out to other resources such as those that include predicted and experimentally validated targets of microRNAs. Last update in 2019	2006 (born in 2004 as microRNA registry)	miRNAs	sequence data, annotation and predicted gene targets	https://doi.org/10.1093/nar/gkj112	http://www.mirbase.org/
EVmiRNA	Contains curated data of 462 miRNA expression profile datasets of EVs in 17 tissues/diseases, by including browse and search functions, as well as data downloading.	2018	miRNAs	Expression matrices and annotations	https://doi.org/10.1093/nar/gky985	http://bioinfo.life.hust.edu.cn/EVmiRNA
EVAtlas	Updated version of EVmirna which includes comprehensive database for expression profiles of 7 types of ncRNAs (miRNA, piRNA, rRNA, tRNA, YRNA, snRNA and snoRNA) in more than 2000 EV samples from 24 human tissues of different conditions (disease and normal). It provides also miRNA/snoRNA/tRNA TCGA expression, miRNA targets, miRNA related drugs, miRNA regulated pathways, miRNA functions, and related publications.	2018	miRNA, piRNA, rRNA, tRNA, YRNA, snRNA and snoRNA	Expression matrices and annotations	https://doi.org/10.1093/nar/gky985	http://bioinfo.life.hust.edu.cn/EVAtlas/#/
ISEV portal	The ISEV is the leading professional society for researchers and scientists involved in the study of microvesicles and exosomes. The online portal allows to access to multiple resources (eg JEV). Currently updated	2011	NA	Online reseources	https://doi.org/10.3402/jev.v1i0.18514	https://www.isev.org/
EVtrack	A database which records experimental parameters in order to improve reproducibility and interpretability. Last update in 2021	2012	NA	Experimental parameters	https://doi.org/10.1038/nmeth.4185	http://www.evtrack.org/
miRandola		2012				

(*continued on next page*)

Table 1 (*continued*)

Name	short description	first year of publication	molecules	type of data	publication	site
	Database of extracellular non-coding RNAs (ncRNAs), including the EVs. The aim of is to collect all the information about ncRNAs that circulate in the blood stream and other body fluids. Last update in 2017		circRNAs, lncRNAs and mRNAs	Literature based features (interaction with other databases)	https://doi.org/10.1093/nar/gkx854	http://mirandola.iit.cnr.it/
exoRBase	Repository of circular RNA (circRNA), long non-coding RNA (lncRNA) and messenger RNA (mRNA) derived from RNA-seq data analyses of human blood exosomes.	2017	circRNAs, lncRNAs and mRNAs	Expression matrices (experimental validation files are included)	https://doi.org/10.1093/nar/gkx891	http://www.exorbase.org/
EVpedia	Integrated database of high-throughput datasets from prokaryotic and eukaryotic extracellular vesicles. An array of tools is integrated for data analysis (eg GO enrichment, network analysis).	2012	proteins, mRNAs miRNAs, lipids	high-throughput datasets	https://doi.org/10.1016/j.semcdb.2015.02.005	http://evpedia.info/
CMEP	Circulating MicroRNA Expression Profiling (CMEP) database for integrating, analyzing and visualizing the large-scale expression profiles of phenotype-specific circulating miRNAs. The CMEP database contains massive datasets that were manually curated from NCBI GEO and the exRNA Atlas, including 66 datasets, 228 subsets and 10 419 samples.	2019	circulating miRNAs	high-throughput datasets	https://doi.org/10.1093/bioinformatics/btz042	http://syslab5.nchu.edu.tw/CMEP/
ExoBCD	Constructed with the combination of robust analysis of four high-throughput datasets, transcriptome validation of 1191 TCGA cases and manual mining of 950 studies. In ExoBCD, approximately 20 900 annotation entries were integrated from 25 external sources and 306 exosomal molecules (49 potential biomarkers and 257 biologically interesting molecules).	2020	mRNAs, miRNAs and lncRNAs	Annotation and curated literature.	https://doi.org/10.1093/bib/bbaa088	https://exobcd.liumwei.org/
R2:HumanBiofluidRNAAtlas Genomics Analysis and Visualization Platform	Atlas of messenger, circular, and small RNA transcriptomes of a comprehensive collection of 20 human biofluids.	2020	shortRNA and mRNA capture	high-throughput datasets	https://doi.org/10.1016/j.celrep.2020.108552	https://hgserver1.amc.nl/cgi-bin/r2/main.cgi?&dscope=HBFRA

patient. The longitudinal analysis of samples deriving from the same individual has the potential to improve sensitivity and reproducibility through the reduction of inter-patient variability (Fig. 1).

Altogether, transcriptomics analysis of patients' circulating EVs may provide valuable information relevant to the originating cancer tissue (including metastasis) and to intercept signals exchanges between tumoral and healthy cells. Analyses that have mainly been focused on small RNAs so far will benefit from the inclusion of long RNAs, since EVs-associated lncRNAs and mRNAs investigation may improve the characterization of cancer-related transcriptional programs.

4. Further directions and opportunities

Based on a recent worldwide survey from the International Society for Extracellular Vesicles (ISEV) [48], nucleic acids represent the EVs-associated molecules with the highest biomarker potential, although their loading on the EVs and the relevant delivery to other cells are still poorly understood [46,150]. For instance, growing bodies of evidence suggest the direct involvement of EVs transcripts in cancer processes, albeit a description of EVs-mediated *in vivo* communication mechanisms have not been fully elucidated yet [151].

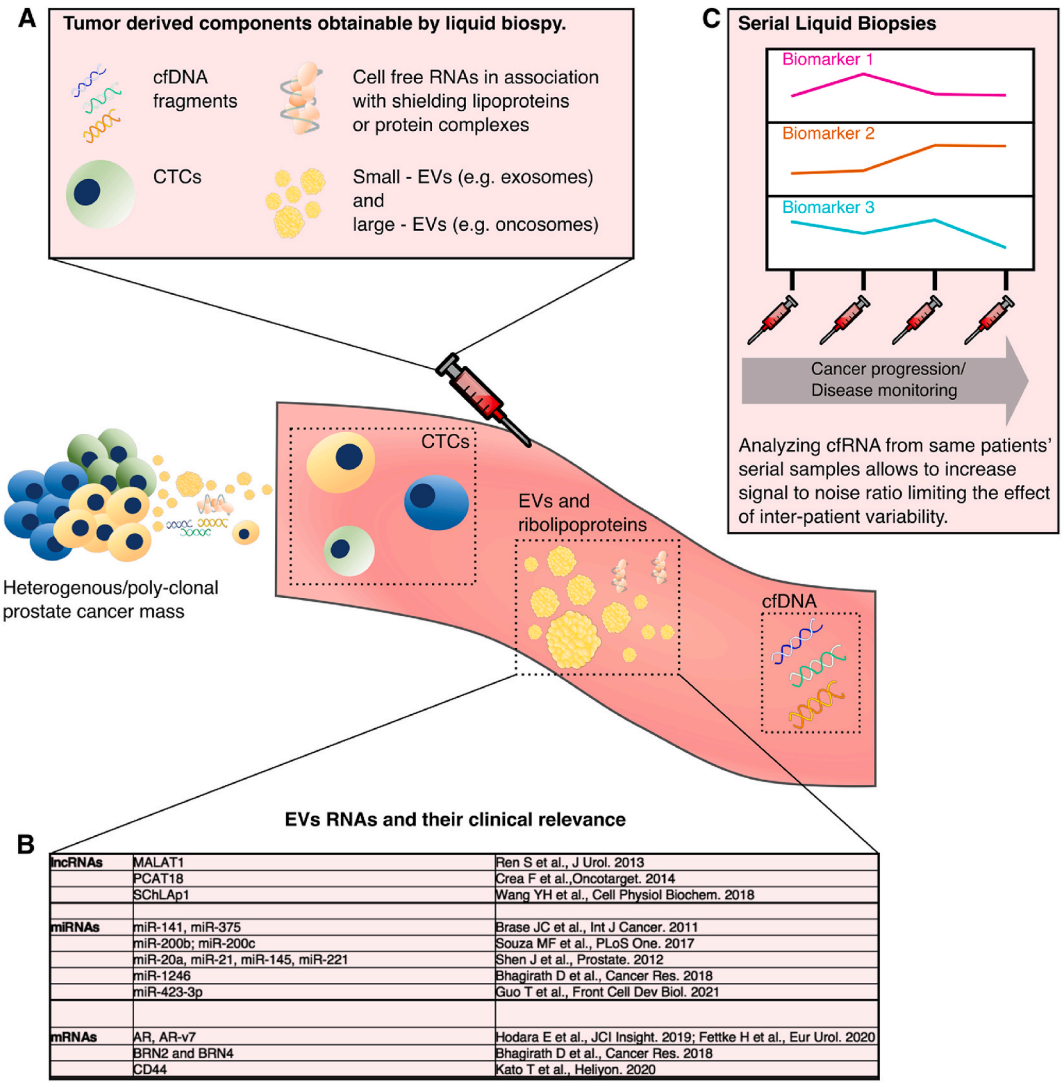
Elucidating the function of the circulating EVs cargo will facilitate the identification of cancer cells-associated molecules and ultimately improve the use of EVs-associated nucleic acids (such as the mRNA and lncRNA) as reliable cancer biomarkers.

However, the lack of the full understanding of the circulating EVs' role in cancer patients does not represent an impediment to the profiling of the EV-RNAs cargo. EVs are currently considered an informative circulating component from body fluids [152] and have been used for the development of multi-analyte blood tests in cancer liquid biopsies [153]. Analysis and integration of multiple circulating components, such as circulating proteins with cfDNA [154] or cfDNA with CTCs, allow precise and sensitive cancer detection [155].

In the future, technological advances in the isolation and characterization of specific EVs subpopulations will improve the capability to differentiate cancer-generated EVs from the high percentage of EVs released by normal cells in the blood or in the urines. While it has been reported that large tumor-derived EVs from CRPC patients can be analyzed by instruments (such as CellSearch) used for CTCs [29], sorting specific subpopulations of small EVs (i.e. exosomes) by flow cytometry is still challenging [28].

Growing opportunities to analyze fluorescent s-EVS will be provided by cutting-edge technologies, such as nano-flow cytometry [156] and imaging flow cytometry (e.g. Imagestream), which have already been tested on large-EVs [157] and exosomes [158,159].

In this perspective, the discovery of cancer-specific markers



lncRNAs	MALAT1	Ren S et al., J Urol. 2013
	PCAT18	Crea F et al.,Oncotarget. 2014
	SChLAp1	Wang YH et al., Cell Physiol Biochem. 2018
miRNAs	miR-141, miR-375	Brase JC et al., Int J Cancer. 2011
	miR-200b; miR-200c	Souza MF et al., PLoS One. 2017
	miR-20a, miR-21, miR-145, miR-221	Shen J et al., Prostate. 2012
	miR-1246	Bhagirath D et al., Cancer Res. 2018
	miR-423-3p	Guo T et al., Front Cell Dev Biol. 2021
mRNAs	AR, AR-v7	Hodara E et al., JCI Insight. 2019; Fettke H et al., Eur Urol. 2020
	BRN2 and BRN4	Bhagirath D et al., Cancer Res. 2018
	CD44	Kato T et al., Heliyon. 2020

Fig. 1. Schematic representation of circulating components as a source of PCa biomarkers from blood-based liquid biopsies.
Molecular and clonal heterogeneity of PCa is here represented by color-coded cells (blue, green, yellow). Tumor mass cells release in the circulation multiple components: CTCs, Extracellular RNAs (exRNAs) that circulate in the blood flow in association with either shielding carriers or small and large EVs; cell free DNA (cfDNA).
A. Legend for the tumor–derived materials included in the blood vessel.
B. A list of clinically relevant exRNAs detected in patients' plasma or serum
C. Analysis of tumor–derived materials from the same patient's serial samples have the potential to be useful for diagnosis and monitoring disease progression during time. Furthermore, the use of serial samples allows increased sensitivity and reproducibility on data analysis by limiting the effect of inter-patient variability.

expressed on the EVs lipidic bilayer will facilitate the identification and quantification of the EVs percentage that is secreted by the primary tumor mass or by the metastasis. For instance, Khanna K. et al. identified STEAP-1 (a protein previously shown to be enriched in prostate tissue) on plasma-isolated EVs from healthy men and PCa patients [45]. STEAP-1 positive–EVs were isolated and quantitated in both cohorts to demonstrate an increase in STEAP-1 marker in presence of PCa [45].

EV-RNAs cargo might be explored similarly to the CTCs-associated intracellular RNA to obtain both genotype and phenotype information associated to cancer cells [160]. Importantly, EVs isolation methods based on specific surface markers will aid in associating the EV-RNAs cargo transcriptional information to a specific tissue.

So far, the identification of cancer specific EVs markers has preferentially been conducted *in vitro*, as demonstrated by Ciardiello C. et al. In this study they showed that the overexpression of alphaV-integrin in large oncosomes isolated from the PCa cell line DU145R80 was able to induce invasion and adhesion in recipient cells [161]. In this respect, proteomics studies on EVs isolated from PCa patients will significantly contribute to the characterization of EVs–associated markers and characterization of tumor–associated EVs populations [162,163].

Being able to sort specific EVs populations by using specific markers will also improve the analysis of the RNA cargo for specific exRNAs classes, such as mitochondrial RNAs (mtRNAs).

Indeed, the mtRNAs represent a potential source of cancer cells-specific metabolic markers because of their peculiar function in controlling cancer cell metabolism [164]. It has already been reported that cells can secrete mitochondrial components into the culture media [165] and that entire mitochondria are transferred between cells by EVs [166]. The analysis of circulating mitochondrial transcripts may represent an innovative approach to test alterations in PCa, a cancer where mutations in metabolic enzymes genes (such as IDH1/2) are known to drive cancer progression [167–169].

Altogether, the studies summarized here demonstrate the concrete possibility to use EV-RNAs biomarkers in PCa and highlight the critical

need of improving technologies and computational approaches to better characterize specific EVs population and their relevant information.

Future work will definitely benefit from novel strategies for sorting and sequencing single EVs populations to facilitate the disentangling of the heterogeneous transcriptional signal obtained from EVs. Furthermore, single-EV-derived RNA sequencing approaches similar to those used for single–cell sequencing will potentially further improve the sensitivity and specificity of transcriptomics data analysis.

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Author contributions section

V.M and Y.C. drafted the manuscript. All authors contributed to the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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